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MOLECULAR BIOLOGY OF ELASTIN AS RELATED TO AGING AND ATHEROSCLEROSIS

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UNDERSTANDING of the structure of elastin and of its physicochemical properties will beyond doubt contribute a great deal to a better understanding of the molecular mechanisms of the aging process of connective tissue. It is true that we have the age of our arteries. This means more or less that we have the age of their elastic fibres. Their change with age shows both slow progressive modifications, such as increase in calcium content, and also to pathological alterations leading eventually to total loss of function due to the atherosclerotic process. In order to gain a better understanding of this pathological processes we first have to understand the normal biology of the arterial wall and of elastic tissue. We would like to sum up briefly some of the most recent results obtained in our laboratory and in some others, over the last few years, and give a general picture of the development of the molecular biology of elastin; that means function and structure of elastin at the molecular level.

PHYSICOCHEMICAL PROPERTIES OF ELASTIN

Elastin is identified by methods which all have in common hydrolytic or strongly denaturing steps which dissolve all the connective tissue proteins except elastin. This methodological definition leaves some doubt about the chemical identity of elastin as isolated and identified by this procedure. The aminoacid composition of elastin obtained by these different procedures is fairly uniform, however, showing that apart from small amounts of impurities, the main component of elastin is a single protein which is considered to be derived from proelastin or tropo-elastin (Partridge, 1969; Sandberg *et al.*, 1969; Robert, 1970).

Table 1 gives a survey of the most frequently used methods and Fig. 1 shows the flowsheet of the procedure we use in order to obtain not only elastin but also some of the other molecular components accompanying elastin, which were also analysed in some detail (Robert *et al.*, 1965). The purification procedure we adopted in the last few years in order to purify elastin uses cold 70 per cent trichloracetic acid for the extraction of collagen and other linked proteins (Robert *et al.*, 1970a). This method is very simple and gives reproducible and reliable results.

All these elastin samples have one common property: their insolubility in hot 0·1 N sodium hydroxide at 100° for 45 min. We found however several years ago that by adding organic solvents, the resistance of elastin to alkaline hydrolysis decreases greatly (Robert and Poullain, 1963). The efficiency of organic solvents to decrease the resistance of elastin to alkaline hydrolysis depends to a great extent on their structure and Table 2

TABLE 1. METHODS USED FOR THE PURIFICATION OF ELASTIN FROM BOVINE LIGAMENTUM NUCHAE OR FROM AORTA. THE FIRST STEPS OF ALL PROCEDURES INVOLVE DELIPIDATION OF THE TISSUE WITH ORGANIC SOLVENTS AND THE EXTRACTION OF DIFFUSIBLE COMPONENTS WITH BUFFER SOLUTIONS SUCH AS 1 M NaCl (Robert and Poullain, 1963; Lowry *et al.*, 1947) OR 1 M $CaCl_2$ (Robert *et al.*, 1965), FOLLOWED BY WASHING. THE WASHED AND ACETON-DRYED TISSUE IS DESIGNATED AS THE "POLYMERIC STROMA". THE FOLLOWING TREATMENTS APPLY TO SUCH "POLYMERIC STROMATA"

No.	Method used to remove foreign proteins and polysaccharides	Reference
1	45 min extraction with 0·1 N NaOH at or about 100°	Lowry *et al.* 1947 Lansing 1952 Banga 1952
2	Repeated autoclaving in water at 15 lbs/in²	Partridge *et al.* 1955
3	Extraction with 88 per cent formic acid	Ayer *et al.* 1958
4	Extraction with mild acids (acetic acid 1 per cent) or autoclaving in 1 per cent acetic acid	Hall 1955
5	Extraction with trypsin and collagenase	Hospelhorn, Fitzpatrick 1961
6	Extraction with 5 M guanidine and dithiothreitol followed by treatment with collagenase	Ross and Bornstein 1969
7	Extraction with 70 per cent trichloracetic acid at + 4°	Robert *et al.* 1970a
8	Serial extractions with 10 per cent $CaCl_2$, 2·7 to 5 per cent TCA at 90° and 8 M urea and 0·1 M mercaptoethanol (see Fig. 1)	Robert *et al.* 1965, 1970

shows in some detail the results of our investigations in this respect (Kornfeld-Poullain and Robert, 1968). It appears that in order to solubilize elastin in the presence of alkali the organic solvents must contain at least 1 polar group and an excess of hydrophobic alkyl residues either in straight or branched chain or in a ring structure. These results suggested the following picture for the alkaline degradation of elastin. Elastin contains tightly packed peptide chains stabilized by strong hydrophobic interactions. These tightly packed peptide chains could have some structural order, as for instance that suggested by the data of Partridge (1969). Both Partridge and Urry (1969) and Gotte *et al.* (1968) recently found evidence for the presence of some conformational order such as α-helices in the structure of soluble alpha elastin, as well as in fibrous elastin. In the presence of an organic solvent the hydrophobic interactions would decrease to a varying extent and the tertiary and quaternary structure of the elastin network would be modified according to the nature and the amount of solvent used. This effect of the organic solvents is completely analogous to the denaturation of globular hydrophilic proteins (Joly, 1965).

Figure 2 shows in a schematic way the effect of organic solvents on elastin structure. The elastic fiber in its normal state is visualized as composed of two types of sub-structures:

(a) The central "amorphous" fiber, shown in the Figure, is composed of the idealized proelastin units, having a more or less spherical conformation. These proelastin units are linked to each other by the cross-links originating from the condensation of α-amino-adipic semi-aldehyde and lysine (Partridge, 1969). The overall structure of this "amorphous" region could be similar to that postulated by Partridge from partition experiments (Partridge, 1967).

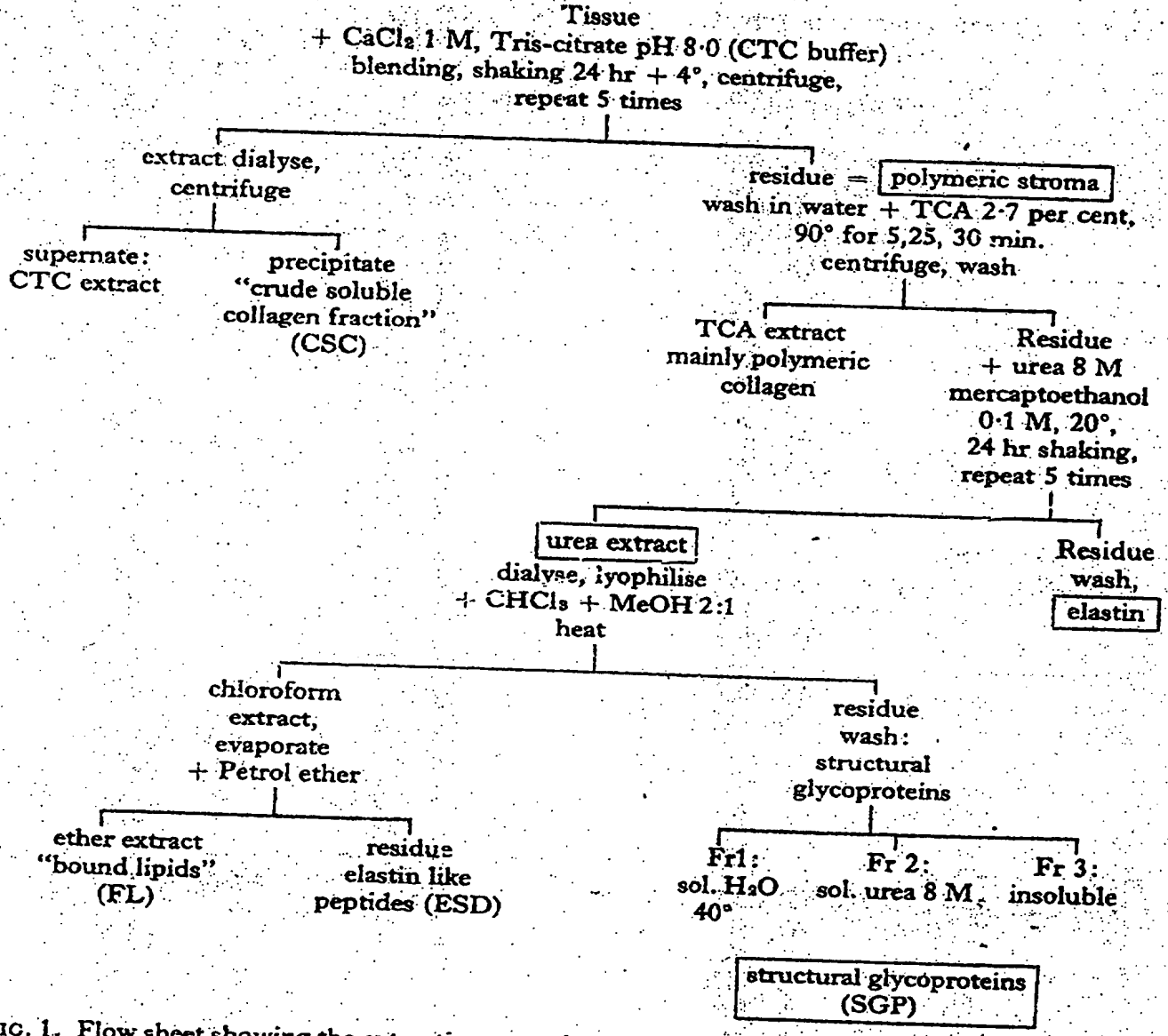

FIG. 1. Flow sheet showing the extraction procedure used to obtain separately polymeric collagen, structural glycoproteins and elastin from aorta and other tissues (Robert *et al.*, 1965; Moczar and Robert, 1970)

(b) The peripheral part of the fiber is rich in "microfibrils" observed and described by several authors (Ross and Bornstein, 1969; Kadar *et al.*, 1969; Haust *et al.*, 1965; Waisman *et al.*, 1969; Cox and O'Dell, 1966). We shall discuss later the relationship of these microfibrils to the chemical composition of elastin.

When an appropriate organic solvent is added as shown in the figure by the *T*-shaped sign, a conformation change will ensue in the proelastin units. This conformation change would be brought about mainly by the disaggregation of some of the hydrophobically stabilized regions due to a close interaction between the apolar aminoacid residues of the proelastin peptide chains and the solvent (Kornfeld-Poullain and Robert, 1968). In their more unfolded ("denatured") state the peptide bonds would be much more susceptible to the attack of hydroxyl ions because of the greater accessibility of these peptide linkages produced by the action of alcohols or other solvents.

TABLE 2. RELATION BETWEEN STRUCTURE OF ORGANIC SOLVENTS AND THEIR "EFFICIENCY" IN INCREASING THE RATE OF ALKALINE DEGRADATION OF ELASTIN. 100 mg FIBROUS ELASTIN WAS INCUBATED FOR 5 hr WITH 10 ml OF A SOLVENT MIXTURE CONTAINING 20 per cent (v/v) WATER AND 80 per cent (v/v) OF AN ORGANIC PHASE COMPOSED OF INCREASING AMOUNTS OF THE ORGANIC SOLVENT TO BE INVESTIGATED AND DECREASING AMOUNTS OF METHANOL (METHANOL'S "EFFICIENCY" IS VERY LOW IN THIS TEST). AFTER 5 hr AT 37° THE AMOUNT OF ELASTIN PEPTIDES PRESENT IN THE SUPERNATE ARE DETERMINED. THE "EFFICIENCY" (ε_{50}) OF THE ORGANIC SOLVENT IS EXPRESSED AS THE INVERSE OF ITS MOLE FRACTION (n_{50}) IN A MIXTURE GIVING 50 PER CENT "SOLUBILIZATION" OF ELASTIN IN THESE CONDITIONS. (After Kornfeld-Poullain and Robert, 1968)

Structural factor	Solvent	Efficiency $\varepsilon_{50} = \frac{1}{n_{50}}$	Observation
Number of CH_2 residues	Aliphalic alcohols (sat. normal)	C_2 2·74 C_3 4·42 C_4 5·62 C_5 7·14	Increases with n-CH_2–
	carbinols	C_2 2·74 C_3 4·42 C_4 5·62	
Configuration of the alkyl chain	Normal and isomeric aliph. alcohols	C_3 4·42 iC_3 5·50 C_4 4·65 iC_4 4·86 IIC_4 5·61 $IIIC_4$ 6·33	Higher for branched chains than for straight chains
Different polar residues S = O –OH C = O –N = –OH heterocycle with 1 O or 1 N or 2 O's	DMSO *i.* prop. OH acetone methylam methanol dioxane morpholine	3·25 6·50 5·35	Efficiency decreases OH or C=O > S=O –N= > –OH 2O > N+O
Ratio of hydrophylic to hydrophobic residues additional polar groups (–OH –O–)	Benzene petrol ether etOH ethylene glycol prop. OH propandiol glycerol prop. OH methyl cellosolve but. OH ethyl cellosolve	0 0 2·74 0 9·25* 0 0 9·25* 1·04* 11·10* 2·30*	Inefficient without polar groups – efficiency decreases with number of OH– efficiency decreases with increasing –O– (*ε_{25})

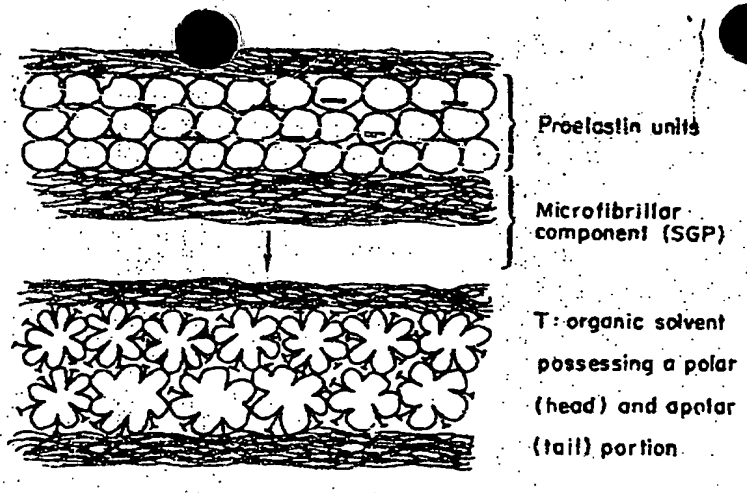

FIG. 2. Model showing schematically the structure of elastin fibres.

The hydrated polar group of these solvents would tend to increase the solvation of the peptide chains. Recent studies carried out in collaboration with Prof. Houtman, Dr. Medema in Delft and Dr. Stack in Bristol using the ^{85}Krypton-method (Robert *et al.*, 1970b) and flow calorimetry with different organic solvents confirm this general view (see Table 3) (Robert *et al.*, 1970b; Stack and Robert, 1970).

TABLE 3. SURFACE AREA MEASUREMENTS ON ELASTIN AND RELATED PROTEINS BY: (1) MEASURING THE ADSORPTION OF ^{85}KRYPTON AND (2) MEASURING THE HEAT OF DESORPTION OF *n*-BUTANOL IN A FLOW COLORIMETER (FROM DATA OBTAINED IN COLLABORATION WITH PROF. HOUTMAN, DR. MEDEMA, DELFT AND DR. M. STACK, BRISTOL). BOTH METHODS WERE CALIBRATED ON A STANDARD TiO_2 SAMPLE. (Robert *et al.*, 1970b; Stack and Robert, 1970)

Sample	Appearant surface area m²/g measured with: ^{85}Kr	*n*-Butanol
Polymeric stroma human aorta	2·8	2·0
Elastin, from human aorta, purified by 0·1 N NaOH	0·8	0·7
Elastin, from bovine ligamentum nuchae purified by guanidine extract.	1·9	1·7
Elastin from bovine ligamentum nuchae, purified by autoclaving	1·0	0·6
Elastin, from bovine ligamentum nuchae purified by TCA-extract.	2·2	3·2
Elastin from bovine ligamentum nuchae, purified by HCOOH extract.	4·5	19·0
Kappa-elastin	0·3	3·2
Keratine	0·1	14

The surface area of elastin samples was measured together with some related protein preparations. When ^{85}Kr was used according to the procedure described by Houtman and Medema, the surface areas obtained varied between 0·1 m^2/g for keratin to 4·5 m^2/g for a formic-acid-purified elastin sample (Robert *et al.*, 1970b). Using flow calorimetry with *n*-butanol as a solvent, the apparent surface values, calculated from the heat of desorption as compared to a standard TiO_2 sample, were sometimes higher than those obtained with ^{85}Kr (Stack and Robert, 1970). This is evident also from the values obtained for the keratin sample, which gives 0·1 m^2/g with ^{85}Kr and much higher apparent surface areas with *n*-butanol and *ter*-butanol. This increase is probably due to the much stronger interactions of *n*-butanol and *ter*-butanol with the peptides than in the case of ^{85}Kr (Kornfeld-Poullain and Robert, 1968) (see Fig. 2). Therefore we can assume the ^{85}Kr values as being closer to the "real" surface of these samples than the values obtained by flow calorimetry. This method, on the other hand, enables a more detailed investigation of these interactions by varying the organic solvent and studying the heat effects produced.

The surface area of elastin as measured by the adsorption of ^{85}Krypton is of the same order of magnitude whether it is measured on purified elastin or in the native fibrous stroma obtained after the elimination of the freely diffusable components by repeated extraction with 1 M sodium chloride (Robert *et al.*, 1970a). These results suggest that elastin is probably directly accessible to small molecules in the arterial wall. Electron-microscopy of elastic fibers in normal and pathological aorta also illustrates these views (Ross and Bornstein, 1969; Kadar *et al.*, 1969; Cox and O'Dell, 1966).

The general picture of the structure of elastin arrived at by these procedures is in agreement with its very high content of aliphatic aminoacids (Gotte *et al.*, 1963; Robert and Poullain, 1966). It also helps to explain the entropic nature of its elasticity (Robert and Poullain, 1966). As a matter of fact, the preponderance of hydrophobic interactions in elastin would exclude most of the water from the inside of the molecular units of the elastin network (tropo- or proelastin units). By traction the conformation of the elastin network could be deformed, rather as it is by the organic solvents, forcing the hydrophobic residues, or at least many of them, in closer contact with water. Water would have to "restructure" around these hydrophobic residues and this procedure would lead to an important decrease of entropy of the system. It would then return spontaneously to its original length by excluding water again from the vicinity of the hydrophobic aminoacid residues.

This hypothesis would also explain why lipids have an unfavorable effect on elastin structure. Lipids could play the role of organic solvents and insert themselves in the hydrophobic regions decreasing the overall stability of the elastic lattice and render it more susceptible to proteolytic attack (see Fig. 2). In agreement with this hypothesis, the addition of increasing amounts of cholesterol to a "low efficiency" organic solvent as methanol (Kornfeld-Poullain and Robert, 1968) significantly increases the speed of alkaline degradation of elastin (Szigeti *et al.*, 1970). Experiments recently carried out in collaboration with Dr. Jacotot and Dr. Szigeti at Créteil University tend to substantiate these results. Radioactive cholesterol administered to rats is partially incorporated into their elastic fibers and can be isolated from their purified elastin, obtained by the method shown in Fig. 1. These findings are also in agreement with histochemical results demonstrating the localization of lipids at the level of elastic fibers in aorta. These results suggested since many years to histochemists the idea that elastin was a lipoprotein (Adams, 1967).

NATURE OF THE SUGAR COMPONENTS OF ELASTIN

It has been known for years that elastin contains some sugar components (Hall, 1955; Loewen, 1965; Gotte *et al.*, 1964). The quality and quantity of the sugar components depend to a great extent on the method used for the purification of elastin. In collaboration with Dr. Moczar, Dr. Szigeti and Mr. Derouette, we recently reinvestigated this problem (Robert *et al.*, 1970a) and Table 4 shows the results of some recent determinations of the hexose and hexosamine content of elastins obtained by the different procedures described in the first section (see Table 1). It can be seen that the hexose and

TABLE 4. CHEMICAL COMPOSITION OF SEVERAL ELASTIN PREPARATIONS OBTAINED FROM BEEF LIGAMENTUM NUCHAE. FROM (Robert *et al.*, 1970a). VALUES NOT CORRECTED FOR MOISTURE AND ASH CONTENT, ON AN "AIR DRY" BASIS

Method of preparation*	Per cent of dry weight			
	Hexoses	Hexosamines	Sialic acid	OH-proline
0·1 N NaOH	0·29	0·31	0·00	1·09
Formic acid	0·49	0·47	0·00	0·89
TCA	0·84	0·50	0·06	1·00
Autoclaving	0·48	0·47	0·00	0·82
Guanidine-extr.†	0·83	0·38	0·04	2·80

* For more details see Table 1.

† Guanidine-extraction not followed by collagenase treatment. Hexoses determined by the anthron procedure, hexosamines according to Elson-Morgan, sialic acid according to Warren and OH-proline according to Bergmann-Loxley.

hexosamine content of these different elastins is quite low but reproducible, between 0·4 and 1 per cent. The hydroxyproline values are also near to those reported previously, except for the guanidine-extracted preparation which was not treated by collagenase and therefore could contain some collagen. The ratio of glucose, galactose, mannose and glucosamine in these elastins is very similar to that found in structural glycoproteins isolated from arterial wall (Moczar and Robert, 1970; Moczar *et al.*, 1970). Therefore we suspected that the sugar component of purified elastin or, at least, part of it may come from the presence of traces of such glycoproteins.

We therefore submitted elastin to the same extraction procedure which is used for the extraction and purification of structural glycoproteins of the arterial wall (Moczar and Robert, 1970). By this procedure which involves repeated extractions with 8 M urea and 0·1 M mercaptoethanol, followed eventually by extraction with 0·1 M Na_2SO_3 (Robert *et al.*, 1970a), up to 80 per cent of the sugar components of elastin could be eliminated together with only about 10 per cent of the peptide components. The extracted glycopeptides again gave analytical values close to those obtained with purified structural glycoproteins. These results suggest that the major part of the sugar components present in elastin are due to the presence of small amounts of structural glycoproteins which were not eliminated during the purification procedure.

Electron microscopical observations of elastin samples prepared by different methods (see Table 1) carried out in collaboration with Dr. Bouissou and Mlle. Fabre in Toulouse,

demonstrated the presence of microfibrils (Robert *et al.*, 1970a), in agreement with the results of Ross and Bornstein (1969). It is interesting to note that the density of the microfibrillar component increases in parallel with the sugar content of these different elastin samples (Table 4). There are much more microfibrils in the TCA-extracted elastin than in the NaOH-extracted elastin sample. Therefore we might tentatively identify the structural glycoprotein components of these purified elastin preparations with the microfibrillar components seen in the electron microscope.

Recent results obtained on the cornea also suggest that structural glycoproteins may be present as microfibrillar structures (Pouliquen *et al.*, 1970; Robert *et al.*, 1970c). Experiments carried out on embryonic cornea (Robert *et al.*, 1969) and rat tail tendon of different ages (Robert *et al.*, 1970d) suggested that the proportion of structural glycoproteins to collagen and/or elastin changes during their evolution. Embryonic tissues appear much richer in structural glycoproteins and the ratio of glycoprotein to fibrillar macromolecules decreases markedly during embyronic development and continues to decrease throughout life. Similar findings were reported by Cleary and Jackson on calf ligamentum nuchae (Cleary *et al.*, 1967). These results tend to suggest that a structural glycoprotein component may play some role in orientation and morphogenesis during the early phases of elastogenesis. These results are in agreement with the observations of electron microscopists (Ross and Bornstein, 1969; Kadar *et al.*, 1969; Haust *et al.*, 1965; Fahrenbach *et al.*, 1966; Waisman *et al.*, 1969; Cox and O'Dell, 1966), who have seen two types of components participating in the early phases of elastogenesis. The first components appears as small, round amorphous droplets staining with phosphotungstic acid; the second component looks like microfibrillar material. Both of these components collaborate in some way in order to form the primitive elastic fibres.

Although these results as well as some metabolic studies to be mentioned later indicate a role for structural glycoproteins in the early phases of elastogenesis it can by no means be inferred that a structural glycoprotein component identified in purified elastin is an integral molecular component of "polymeric elastin". It can indeed be eliminated by methods which do not rupture covalent bonds (other than S–S-bonds) and it may not have a pre-eminent role in the quaternary structure of adult elastin. This point, however has to be investigated further.

Whatever the functional significance of this association between structural glycoprotein and collagen or elastin may be, the ratio of these components changes with age and may be considered as one of the chemical parameters of the aging of connective tissue.

THE STRUCTURAL GLYCOPROTEINS OF THE ARTERIAL WALL

Several authors reported the presence of glycoproteins in the arterial wall (Gotte *et al.*, 1964; Moczar and Robert, 1970; Moczar *et al.*, 1970; Berenson and Fishkin, 1962; Barnes and Partridge, 1968). The method we proposed (Fig. 1) enabled us to extract a significant portion of these glycoproteins from the polymeric stroma with urea (Moczar and Robert, 1970). The nature of these glycoproteins was investigated in detail in collaboration with Dr. and Mrs. Moczar (Moczar and Robert, 1970; Moczar *et al.*, 1970). The crude urea extract of the arterial wall is further purified by the procedure outlined in Fig. 1. About 60 per cent of the total dry weight extracted by urea, are present in the final delipidated

residue. This residue can be divided further in 3 fractions having different solubilities: the first fraction is soluble at 40° in water, the second only in 8 M urea and the third remained insoluble. The analytical results (composition of sugars and aminoacids) obtained on these three fractions are nearly identical and it appears possible that they might differ only in the degree of crosslinking. The fraction soluble in petrol-ether contains different lipids and phospholipids which can be considered as the "bund lipids" of the arterial wall. The hydrosoluble fraction of the chloroform-methanol extract (ESD, see Fig. 1) contains peptides and gives an aminoacid analysis similar to that of elastin but does not contain detectable amounts of desmosine or isodesmosine (Moczar and Robert, 1970).

TABLE 5. CHEMICAL COMPOSITION OF A PURIFIED STRUCTURAL GLYCOPROTEIN FRACTION FROM PIG AORTA (Moczar and Robert, 1970)

	Per cent of dry weight
Hexoses (orcinol)	2·1
Hexosamines (Elson-Morgan)	2·86
	residue/1000 residues
OH-Lys	0·83
Lys	36·6
His	13·2
Arg	33·5
OH-pro	0
Asp	70·3
Thr	35·0
Ser	33·6
Pro	171·3
Glu	90·4
Gly	124·7
Ala	96·7
Val	60·6
Cyst	0·5
Met	3·3
i-Leu	36·4
Leu	75·2
Tyr	29·6
phe	37·7

The chemical composition of the structural glycoproteins purified as described (Fig. 1) is given in Table 5. Recent experiments carried out on the glycopeptides obtained by pronase digestion of this structural glycoprotein and also of the polymeric stroma of aorta revealed a great heterogeneity of the oligosaccharide side chains composing these glycopeptides (Moczar *et al.*, 1970). Every one of the oligosaccharides obtained by gel filtration followed by high voltage electrophoresis contains glucose, galactose, mannose, glucosamine, fucose and sialic acid in varying ratios. Two dimensional thin-layer chromatography of partial hydrolysates of the glycopeptides revealed differences in the detailed structure of the oligosaccharide components obtained from different species (human, pig, horse, sheep). As these structural glycoproteins are strongly antigenic their organ and species specificity (Robert *et al.*, 1968) may be related to the microheterogeneity and species-dependent variations of these glycopeptides.

BIOSYNTHESIS OF ELASTIN AND OF THE STRUCTURAL GLYCOPROTEINS

We mentioned already some of the morphological observations concerning the correlation between the biosynthesis of structural glycoproteins and of proelastin in the early phases of elastogenesis. In order to gain a better understanding of the process of repression of elastin biosynthesis in adult organisms we reinvestigated the incorporation of labelled aminoacids and sugars in aorta proteins using adult rabbits, both normal and atherosclerotic (Robert *et al.*, 1970). Some rabbits received repeated injections of structural glycoprotein preparations obtained from human or pig aorta, others were injected with kappa-elastin prepared from the same sources, together with complete Freund's adjuvant. Another group of rabbits were fed with a cholesterol-rich diet and a third group received only Freund's complete adjuvant. After two months of treatment the rabbits were sacrificed and their aortas cut in small pieces and incubated in a Krebs–Ringer phosphate medium with ^{14}C-lysine. The aorta slices were then washed with an excess of cold lysine and three times with Krebs–Ringer phosphate and extracted according to the schema shown in Fig. 1. All the extracts contained non-dialysable radioactivity.

TABLE 6. *In vitro* INCORPORATION OF ^{14}C-LYSINE IN THE POLYMERIC STROMA OF RABBIT AORTA. THE AORTAS OF RABBITS, PRETREATED AS INDICATED, WERE CUT IN SMALL SLICES AND INCUBATED IN A KREBS–RINGER–PO_4 MEDIUM WITH 5 μC OF ^{14}C-LYSINE. AFTER WASHING THE AORTAS WERE EXTRACTED AND FRACTIONATED BY THE METHOD SHOWN IN FIG. 1 AND THE RADIOACTIVITY OF THE FRACTIONS DETERMINED BY SCINTILLATION-COUNTING. RESULTS ARE GIVEN AS THE AVERAGE OF SEVERAL DETERMINATIONS, IN dis/min/mg PROTEIN (DATA FROM Robert *et al.*, 1970e)

Pretreatment of the Rabbits	No. of rabbits	Radioactivity in fraction* dis/min/mg prot. TCA-extract (polymeric collagen)	SGP† (Urea-soluble fraction)	ESD‡	Final§ residue
Freund's adjuvant	4	393	4870	1860	630
Immunized with structural glycoproteins of human aorta in Freund's adjuvant	3	538	2890	1100	1770
Cholesterol fed	3	393	2830	1630	590

* For the designation and origin of fractions (see Fig. 1).

† Purified, delipidated urea soluble structural glycoprotein fraction (see Fig. 1).

‡ Hydrosoluble fraction of the chloroform-methanol-extract, contains an elastin like protein (see Fig. 1).

§ Corresponds to elastin (see Fig. 1).

Table 6 shows the distribution of radioactivity in several fractions. The diffusable and calcium chloride extractable fraction contain a considerable specific activity (not shown in Table 6). The polymeric stroma also contains a significant amount of the label. The trichloracetic acid extract containing mainly the polymeric collagen contains about 400–600 dis/min/mg proteins. The structural glycoprotein fraction (purified urea extract) contains a significantly higher radioactivity up to several thousand dis/min/mg.

The final residue (elastin) contains also some radioactivity (~ 600–1800 dis/min/mg). The activity of this elastin fraction increases significantly in those rabbit aortas which have been severely attacked by the atherosclerotic process.

The ures extract in these experiments was fractionated according to the flowsheet shown in Fig. 1, and the data shown in Table 6 give the radioactivity obtained in these purified fractions. A small fraction of the total radioactivity is present in an as yet unidentified form in the petrol-ether soluble lipid extract (FL-fraction, see Fig. 1). The water soluble fraction of the chloroform-methanol extract (ESD-fraction) containing peptides with an aminoacid composition similar to that of non-cross-linked elastin have a significantly higher label than the polymeric elastin fraction. Although the real significance of these findings is as yet unknown, it might suggest the possibility of a partial neosynthesis of elastin at least in some of the more severely atherosclerotic aortas. The 3 different forms (water soluble, urea soluble, insoluble) of the purified structural glycoprotein fractions contain nearly identical specific activities (only the urea soluble is shown in Table 6). This identity of labelling confirms our suggestions that these 3 physicochemically distinct forms differ only in the degree of cross-linking and are synthetized from the same pool of precursors. Further studies are in hand in our laboratory in order to establish the exact nature of the labelled products present in these elastin preparations and their relation to a possible derepression of elastin neosynthesis in atherosclerotic aortas.

IMMUNOCHEMICAL PROPERTIES OF ELASTIN

Some years ago we demonstrated that kappa-elastin obtained by alcoholic potassium hydroxide hydrolysis of fibrous elastin was antigenic in rabbits (Robert *et al.*, 1963). We also showed in collaboration with Dr. Stein and Drs. Crouzet and Camus that normal as well as pathological human sera contain antibodies reacting with red cells coated with such soluble elastin preparations (Stein *et al.*, 1965; Crouzet *et al.*, 1970).

We found a correlation between the pathological state of the patients and the titer of their anti-elastin antibodies. These results interpreted in favor of an immunological theory of atherosclerosis are described in more detail elsewhere (Robert *et al.*, 1968; Robert *et al.*, 1966). It was interesting to notice in these studies that the anti-elastin antibodies appeared only at a certain age (~ 20) and decrease again to very low levels after 70 to 80 years of age (Stein *et al.*, 1965).

Figure 3 reproduced from our paper in Nature shows this age dependence of the anti-elastin antibodies determined in human sera. Using the indirect Coons technic, it also

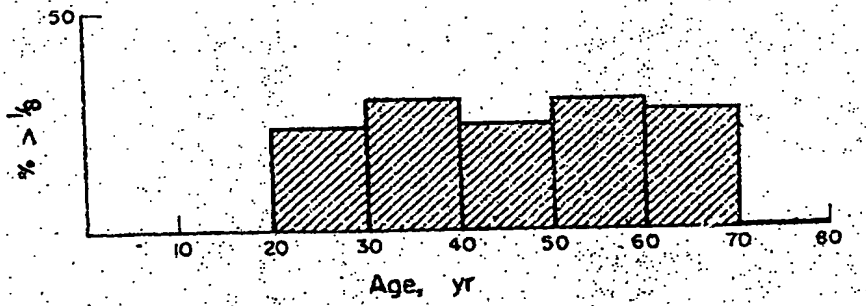

FIG. 3. Anti-elastin antibodies in human sera as a function of the age of the donor (from Stein *et al*, *Nature*, 1965). Ordinates: number of individuals (in per cent of total) having a hemagglutination titer with elastin higher than $\frac{1}{8}$.

could be demonstrated that gamma-globulins were present in atherosclerotic lesions of human aorta at the level of degrading elastic fibers. Figure 4 shows such a section obtained from an advancing human atherosclerotic lesion. It can be seen that the rhodamine stained anti-human gamma-globulin localizes just at the level of the dissociating, degrading elastic fibers.

In agreement with these results, atherosclerotic lesions could be produced in rabbits by prolonged immunization with soluble elastin peptides (kappa–elastin) prepared from human or pig aorta elastin and also with the structural glycoprotein component of the aorta (Robert *et al.*, 1968; Robert *et al.*, 1966; Robert and Robert, 1969). Figure 5 shows such a lesion obtained by treating rabbits for 4 months with kappa–elastin preparations. The histological and electronmicroscopic studies of these aortas carried out by Dr. Grosgogeat, Dr. Reverdi and their colleagues, revealed a very strong degradation of the elastic fibers starting with the lamina elastica interna and proceeding to the media (Grosgogeat *et al.*, 1970).

These experimental results tend to substantiate the immunological hypothesis proposed several years ago and which is shown in some detail in Fig. 6. The experimental evidence confirming most of the phases of this schema were recently summarized (Robert and Robert, 1969).

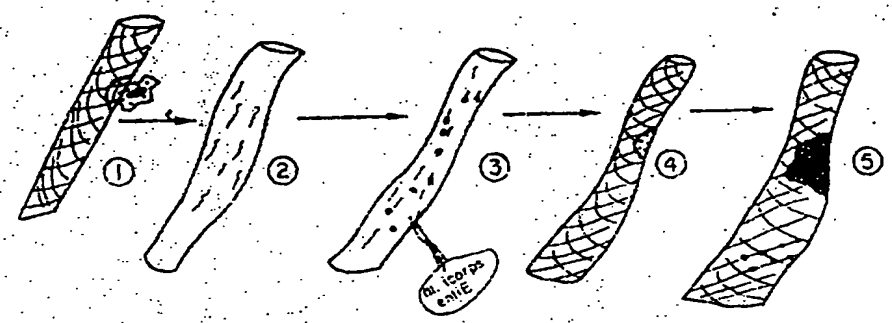

FIG. 6. Figure showing the successive steps of the immunological hypothesis of atherosclerosis (from Robert *et al.*, 1966).

No. 1: localized erosion of the wall (cathespins, elastases, etc.).
No. 2: liberation of soluble peptides from the polymeric stroma.
No. 3: formation of auto-antibodies (anti-elastin, anti-structural glycoprotein).
No. 4: fixation of antibodies in the eroded lesion, on the damaged elastic fibres.
No. 5: progression of the erosion by complement fixation, further elastase action due to antigen antibody triggered liberation of platelet enzymes, lipid and calcium deposition.

Experiments carried out in collaboration with Dr. Caen and Legrand showed at least one possibility for the initial phase of degradation of elastic fibers. We could isolate from human blood platelets a protease which attacks elastin quite efficiently (Robert *et al.*, 1969). Using 125-iodine or 131-iodine labelled elastin we could show that this elastolytic protease is released from blood platelets in the presence of collagen, ADP, or adrenaline (Robert *et al.*, 1970f; Legrand and Robert, 1970). It can be even more efficiently extracted by sonication or by treating human blood platelets with Triton-X-100. The elastolytic protease could be purified from such a Triton extract and separated from the main cathepsin fraction (Legrand and Robert, 0000). The presence of this enzyme in blood platelets suggests that they may be involved in the degradation of the elastic tissue in aorta by a procedure different from that suggested by the classical thrombogenic theory.

FIG. 4. Demonstration by immunofluorescence of the presence of gamma-globulins in atherosclerotic lesions of human aorta. At the arrow rhodamine labelled anti-human gamma-globulin (goat-serum) fixed at degrading elastic fibres (Robert and Robert, 1969 and inedit results).

FIG. 5. Severe atheromatous lesions obtained in rabbits after 6 months treatment with kappa-elastin prepared from human aorta in complete Freund adjuvant (Robert *et al.*, 1968; Robert and Robert, 1969 and inedit results).

(facing p. 350)

This platelet enzyme could be "injected" directly in the vessel wall by adhering platelets, or it could be released slowly in blood plasma and account in part at least for the presence of elastolytic protease described many years ago by Hall (1966), by Loewen (1963, 1969) and by Rinderknecht *et al.* (1968) in human blood serum. Further experiments are needed to confirm the role of this elastolytic platelet protease in the degradation of elastic tissue and of the role of serum elastase inhibitors on the action of this protease.

CONCLUSIONS

Experiments carried out over the last years in several laboratories lead to the following picture of elastogenesis (Fig. 7). At the early embryonic stages of elastogenesis, fibroblasts or other more differentiated cells such as smooth muscle cells synthetize both a proelastin (or tropoelastin) probably similar to that recently isolated by Sandberg *et al.* (1969), and structural glycoprotein (Robert *et al.*, 1965; Moczar and Robert, 1970).

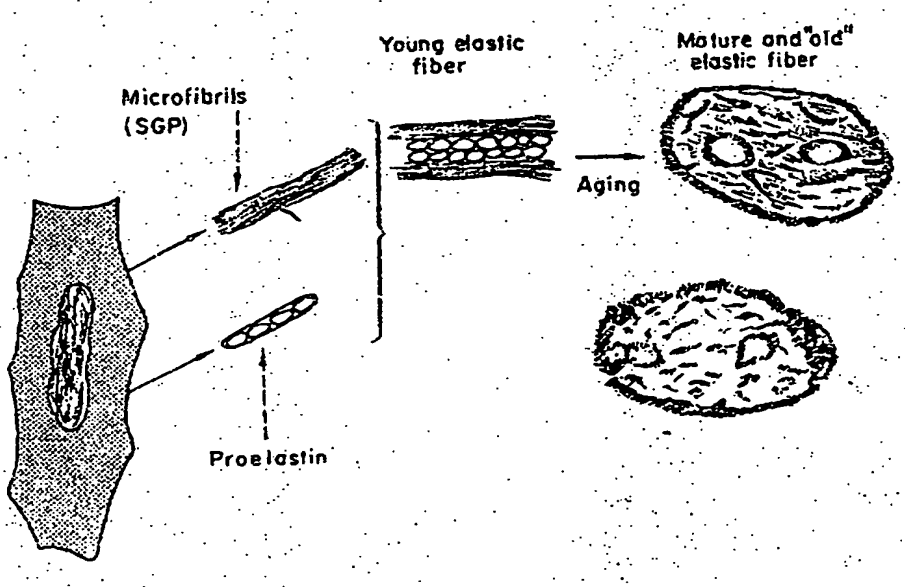

FIG. 7. Schematic drawing showing the successive steps of elastogenesis and "aging" of elastic fibers.

This first appears under the electronmicroscope as amorphous droplets and the second as a microfibrillar material. These two structures cooperate in an as yet un-understood manner to form the primitive elastic fibrils. With maturation and aging the glycoprotein component significantly decreases and eventually disappears. Adult elastic fibers would then resemble the structures proposed by Partridge and ourselves and would contain the original tropoelastin units cross-linked by the desmosine, isodesmosine, lysinorleucine and other less well identified cross-linking aminoacids (Partridge, 1969; Robert, 1970) (see Fig. 2). The tertiary and quaternary structure of these elastic fibers would depend to a great extent on hydrophobic interactions (Kornfeld-Poullain and Robert, 1968). The presence of lipids or organic solvents would have a denaturing effect, decreasing the overall stability and resistance of this elastic network (see Fig. 2). This mechanism may explain in part, at least, the deleterious effect of lipid accumulation in the arterial wall.

The age dependent modifications of elastin are as yet not very well understood. Some of the data accumulated through the work of several investigators were already reviewed here by Hall (1968). Our recent work adds to these mechanisms at least one: the immunochemical procedure by which anti-elastin and other antibodies could contribute to a localized and accelerated degradation of the elastic fibers. This effect of the anti-elastin antibodies could be achieved through different mechanisms. One of them would be the fixation of complement and the activation of complement dependent degrading enzymes partially originating from the lysosomes. Another mechanism would be the facilitation of platelet clumping through the formation of antigen-antibody complexes. Platelets could then release a protease, described recently, which is capable of attacking elastic fibres (Robert *et al.*, 1969). Through such a mechanism a localized degradation of elastin could be obtained similar to that seen in pathological atherosclerotic lesions. This mechanism would also be in agreement with the immunological theory of aging proposed by Walford (1969).

Our recent experiments on ^{14}C-lysine incorporation in elastin and structural glycoproteins of aorta suggest that a derepression of elastin biosynthesis would be possible at least in pathological conditions. When the mechanism of the repression is better understood, it should be possible to induce a neosynthesis of normal elastic fibers through the action of some therapeutic agents.

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Summary—The authors consider in some detail the structure of elastin at the molecular level as related to its normal function and to the modifications accompanying aging and atherosclerosis.

(1) The peculiar *physicochemical properties* of elastin can be best explained by assuming the prevalence of hydrophobic interactions as the main stabilizing forces of its tertiary and quaternary structure. Detailed investigation of the acceleration of alkalin hydrolysis of elastin by a variety of organic solvents confirms this assumption (Kornfeld-Poullain and Robert, 1968). It is postulated that lipidic substances do share with organic solvents their affinity for elastin and may accelerate its "unfolding" and degradation through such "denaturing" effect.

Elastin surface are [redacted]surements with ^{85}Kr and by flow cal[redacted]etry confirmed the direct accessibi[redacted] of elastin in native stroma of aorta [redacted] small molecules (Robert *et al.*, 1970b; Stack and Robert, 1970).

(2) The study of the *sugar components of elastin* suggested the presence of structural glycoproteins (SGP) as "impurities" in all purified elastin samples investigated. These SGP-components are very probably identical with the "microfibrils" seen on electron-micrograms of elastin.

(3) *In vitro incorporation of ^{14}C-lysine* were carried out in aortas of rabbits immunized with purified elastin and SGP or fed on cholesterol rich diet. A strong labelling of the SGP-fraction was obtained and a significant labelling of the polymeric collagen and elastin fractions also. Incorporation was higher in strongly atheromatous aortas. It is concluded that at least a partial "derepression" of elastin synthesis may occur in such conditions.

(4) *The antigenic properties* of elastin can be ascribed also to the presence of both components, polymerized pro- or tropoelastin and SGP-s (microfibrils). The presence of anti-elastin antibodies in human sera was described (Stein *et al.*, 1965; Crouzet *et al.*, 1970). These antibodies appear at about 20 yr of age and decrease or disappear at 70–80 yr. The lower titres found in severely atheromatous sera was ascribed to the adsorbtion of these antibodies to degrading elastic fibers in the aorta and elsewhere. Arguments in favor of our immunological theory of atheromatosis are discussed (see Fig. 6) in the light of recent experiments. Such recent findings are the production of atheromatosis in rabbits immunized with elastin or aorta SGP-preparations and the isolation of a platelet protease capable of attacking elastin (Robert *et al.*, 1969).

Résumé—Les auteurs étudient la structure de l'élastine à l'échelle moléculaire pour dégager les rapports entre celle-ci et sa fonction normale d'une part et ses modifications produites par l'âge et par des conditions pathologiques d'autre part.

(1) Les propriétés physicochimiques particulières de l'élastine peuvent être le mieux expliquées par l'existence de fortes intéractions hydrophobes stabilisant la structure tertiaire et quaternaire. L'étude détaillée de l'accélération de l'hydrolyse alcaline de l'élastine par certains solvents organiques a confirmé ces vues (Kornfeld-Poullain and Robert, 1968). Un effet "déstabilisant" analogue pourrait être obtenu par l'accumulation des lipides dans le tissu élastique qui pourrait à l'instar des solvents organiques, accélérer la dégradation de l'élastine. Des mesures de la surface de l'élastine purifiée et du stroma natif à l'aide du ^{85}Kr et par microcalorimétrie a flux continu ont confirmé l'accessibilité directe de l'élastine au seine du tissu élastique à des petites molécules (Robert *et al.*, 1970b; Stack and Robert, 1970).

(2) L'étude de la nature des composés glucidiques de l'élastine ont suggéré la présence de Glycoprotéines de Structure (GPS) comme "impuretés" dans tous les échantillons d'élastine purifieé qui' ont été examinés. Ces composants GPS paraissent être identiques avec les "microfibrilles" détectées par microscopie électronique dans ces mêmes échantillons.

(3) L'étude de l'incorporation de la lysine-^{14}C dans les aortes de lapins immunisés avec l'élastine ou avec les GPS, ou nourris sur un régime riche en cholestérol a révélé un très fort marquage du collagène et de l'élastine polymériques. Cette incorporation a été plus forte dans les aortes porteuses de lésions étendues. Une "dérépression" partielle de la synthèse de l'élastine paraît donc probable dans ces conditions.

(4) Les propriétés antigéniques de l'élastine peuvent en principe être attribuées aux deux composants décelés: la pro- ou tropoélastine et les GPS ou "microfibrilles". La présence d'anticorps antiélastine dans le sérum humain a été signalée (Stein *et al.*, 1965; Crouzet *et al.*, 1970). Ces anticorps apparaissent vers l'âge de 20 ans

et disparaissent vers 70–80 ans. Les titres plus faibles des sérums de malades athéroscléreux peuvent être attribués à l'adsorption des anticorps sur les fibres élastiques dégradées dans la paroi artérielle. Les arguments en faveur de notre théorie immunologique de l'athérosclérose sont discutés (voir Fig. 6) à la lumière de résultats récents. De tels résultats sont: la production d'athéromatose chez les lapins immunisés avec l'élastine ou avec les GPS de l'aorte ainsi que l'isolement à partir de plaquettes humaines d'une protéase capable d'attaquer l'élastine (Robert *et al.*, 1969).

Zusammenfassung—Die Verfasser betrachten die Einzelheiten der molekularen Struktur des Elastins in Beziehung zu der normalen Funktion und zu Modifikationen, welche mit Altern und Atherosklerose zusammenhängen.

(1) Die besonderen *physikalisch-chemischen Eigenschaften* des Elastins können am besten mit der Annahme erklärt werden, daß die zahlreichen hydrophoben Wechselwirkungen die wesentlichen Stabilisationskräfte der Tertiär- und Quartärstruktur darstellen. Eingehende Untersuchungen der Beschleunigung der alkalischen Hydrolyse von Elastin durch verschiedene organische Lösungsmittel bestätigen diese Annahme (Kornfeld-Poullain and Robert, 1968). Es wird postuliert, daß Lipidstoffe mit den organischen Lösungsmitteln ihre Affinität für Elastin teilen und das "Entfalten" und den Abbau durch einen solchen "Denaturierungseffekt" beschleunigen. Messungen der Elastinoberfläche mit ^{85}Kr und durch Fluß-Kalorimetrie bestätigten die direkte Zugänglichkeit des Elastins im nativen Stroma der Aorta für kleine Moleküle (Robert *et al.*, 1970b; Stack and Robert, 1970).

(2) Die Untersuchung der *Kohlenhydratkomponenten des Elastins* wies auf die Gegenwart von Struktur-Glykoprotein (SGP) als "Verunreinigung" in allen gereinigten Elastinpräparaten hin. Diese SGP-Komponenten sind wharscheinlich mit den "Mikrofibrillen" identisch, welche man auf elektronenoptischen Bildern des Elastins sieht.

(3) *In-vitro-Einbaustudien von ^{14}C-Lysin* in Aorten von Kaninchen, welche mit gereinigtem Elastin und SGP immunisiert waren oder cholesterinreich ernährt waren, wurden durchgeführt. Die SGP-Fraktion wurde stark markiert, eine beträchtliche Markierung fand sich auch im polymeren Kollagen und in Elastinfraktionen. Der Einbau war bei stark atheromatösen Aorten höher. Es wird geschlossen, daß unter solchen Bedingungen wenigstens teilweise eine "Derepression" der Elastinsynthese auftritt.

(4) Die *Antigeneigenschaften* des Elastins können auch der Gegenwart von beiden Komponenten, polymerisiertem Pro- oder Tropoelastin und SGP-s (Mikrofibrillen), zugeschrieben werden. Anti-Elastin-Antikörper im menschlichen Serum wurden beschrieben (Stein *et al.*, 1965; Crouzet *et al.*, 1970). Diese Antikörper erscheinen im Alter von etwa 20 Jahren und nehmen ab oder verschwinden völlig im Alter von 70–80 Jahren. Die erniedrigten Titer bei hochatheromatösen Sera wurde der Adsorption dieser Antikörper an sich abbauende Elastinfasern in Aorta und anderen Stellen zugeordnet. Argumente, welche unsere immunologische Theorie der Atheromatose unterstützen, werden im Lichte neuer Untersuchungen diskutiert (siehe Abb. 6). Neue Befunde sind die Erzeugung einer Atheromatose bei Kaninchen, welche mit Elastin oder Aorten-SGP-Präparaten immunisiert wurden, und die Isolierung einer Thrombozytenelastase, welche Elastin angreift (Robert *et al.*, 1968).

Резюме—Авторы обсуждают детально структуру эластина на молекулярном уровне по отношению к его нормальной функции и к изменениям, сопровождающим старение и атеросклероз.

(1) Своеобразные *физико-химические свойства* эластина лучше всего объясняются, если допустить, что преобладание гидрофобных реакций является главной стабилизирующей силой его третичной и четвертичной структуры. Это предположение подтверждается подробным исследованием ускорения щелочного гидролиза эластина при воздействии различных органических растворителей, (Kornfeld-Pollain and Robert, 1968). Допускается, что липидные вещества разделяют с органическими растворителями сродство к эластину и способны ускорять его "развертывание" и деградацию, благодаря такому действию "денатурации". Измерения поверхности эластина при помощи ^{85}Kя и калометрического измерения текуести подтвердили подходмость микромолекул к эластину в нативной строме аорты (Robert *et al.*, 1970b; Stack and Robert, 1970).

(2) Исследование *сахарных компонентов эластина* указывало на присутствие во всех исследованных очищенных образцах эластина структуральных гликопротеинов (СГП) в качестве "загрязнений". Эти компоненты СГП по всей вероятности тождественны с "микрофибриллами", которые обнаруживаются в электронных микрограмах эластина.

(3) *Включение ^{14}C-лизина in vitro* проводилось в аортах кроликов, иммунизированных очищенным эластином и СГП или же кормленных диетой богатой холестерином. Обнаружилось сильное мечение жракции СГП, а также заметное мечение полимерных фракций коллагена и эластина. Внедрение было выше в сильно атероматозных аортах. Делается заключение, что при этих условиях происходит по крайней мерс частичная "де-репрессия" синтеза эластина.

(4) *Антигенные свойства* эластина можно тоже приписать присутствию обоих компонентов: полимеризированному про- или тропо-эластину и СГП (микрофибриллы). Было описано присутствие анти-эластиновых антител в человеческих сыворотках (Stein *et al.*, 1965; Crouzet *et al.*, 1970). Эти антитела появляются приблизительно в возрасте 20 лет и снижаются или исчезаютк 70–80 годам. Низкие титры, обнаруженные в сильно атероматозных сыворотках, приписываются абсорбации этих антител к деградирующимся эластическим волокнам в аорте и в других местах. Аргументы в пользу нашей иммунологической теории атероматоза (см.рис.6) обсуждаются в свете новых экспериментов. К таким недавним открытиям относятся воспроизведение атероматоза у кроликов, иммунизированных эластином или препаратами СГП из аорты и выделение тромбоцитной протеазы, способной нападать на эластин (Robert *et al.*, 1968).